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The Estimation of Serum Guanosine Deaminase Activity in Liver Disease

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Summary: An assay for the estimation of guanosine deaminase is described. The method employs guanosine as substrate and after incubation of serum and substrate at 22 °C for 18 h the ammonia liberated is estimated using the *Berthelot* reaction. Absorbance is measured at 625 nm and the catalytic activity read from a standard curve obtained using ammonia standards. The method provides reproducible measurement of serum guanosine deaminase. The results obtained using 'normal' sera have been used to calculate the 'normal range' for the enzyme in serum. Preliminary results suggest that guanosine deaminase is increased in hepatitis and in patients with liver metastases but normal in all other liver diseases including cirrhosis and obstructive jaundice.

Bestimmung von Guanosindesaminase im Serum bei Lebererkrankungen

Zusammenfassung: Eine Methode zur Bestimmung von Guanosindesaminase wird beschrieben. Serum wird mit Guanosin als Substrat inkubiert und nach 18 h bei 22 °C wird das freigesetzte Ammoniak mit der *Berthelot*-Reaktion bestimmt. Die Absorption wird bei 625 nm gemessen und die katalytische Aktivität aus einer mit Ammoniak-Standards erstellten Standardkurve ermittelt. Die mit „Normal"-Seren erhaltenen Ergebnisse wurden zur Berechnung des „Normal"-Bereichs für das Enzym im Serum verwendet. Vorläufige Ergebnisse weisen darauf hin, daß Guanosindesaminase bei Hepatitis und bei Patienten mit Lebermetastasen erhöht, bei allen anderen Erkrankungen der Leber, eingeschlossen Cirrhose und Verschlußikterus, jedoch normal ist.

Introduction

The estimation of dCMP deaminase (EC 3.5.4.12) and cytidine deaminase (EC 3.5.4.5) in pregnancy serum have become important parameters in the early detection of pre-eclamptic toxaemia (1), intrauterine death and viral hepatitis during pregnancy. Both enzymes have been found to be within normal limits in essential hypertension (2).

The clinical value of estimating dCMP deaminase in pregnancy serum has also been reported by *Szekely* et al. (3).

During our investigation into the clinical usefulness of estimating enzymes involved in DNA metabolism during pregnancy it became increasingly clear that dCMP deaminase and cytidine deaminase were also increased in certain liver diseases. Furthermore the activity of guanosine deaminase (EC 3.5.4.15) which is an interconverting enzyme involved in DNA metabolism was increased in activity in liver diseases.

Guanosine deaminase was found to differ from dCMP deaminase and cytidine deaminase in that it was not raised in pre-eclamptic toxaemia or intrauterine death but was valuable in discriminating between certain groups of liver diseases. Normal serum guanosine deaminase catalytic activity concentration was found in alcoholic and cardiac cirrhosis, obstructive jaundice, drug induced cholestatic jaundice and acute cholecystitis with raised values in infective hepatitis and in liver metastases. The enzyme therefore provides a new means of differentiating liver diseases. At present no test of liver function is capable of discriminating between various liver disorders.

However no method is available for the routine clinical determination of serum guanosine deaminase catalytic activity concentration. We have therefore developed a new and sensitive method for the estimation of guanosine deaminase in serum. With this method the substrate guanosine is converted to xanthosine and ammonia. The ammonia liberated is estimated using the *Berthelot* reaction without protein precipitation.

Materials and Methods

Reagents

The substrate guanosine was purchased from Sigma Chemical Co. All other reagents were purchased from British Drug Houses Ltd.

The phosphate buffer, pH 6.8; 0.15 mol/l was prepared by the addition of 53.4 ml of 0.15 mol/l KH_2PO_4 to 46.6 ml of 0.15 mol/l Na_2HPO_4. 80 mg of guanosine was suspended in 10 ml of distilled water (28.2 mmol/l) and this stock solution was diluted 1 in 2 with 0.15 mol/l phosphate buffer. Both substrate solutions were stable for 48 hours at room temperature.

The phenol reagent was prepared by dissolving 10 g of phenol and 50 mg sodium nitroprusside in 1 l of distilled water. For the hypochlorite reagent 5 g sodium hydroxide and 21.3 g disodium hydrogen orthophosphate were dissolved in approximately 800 ml of distilled water to which was added 14 ml of a 100–140 g/l solution of sodium hypochlorite and the volume made up to 1 l. Both the phenol and the hypochlorite solutions were stable for one week at 22 °C.

The stock ammonia standard contained 7.13 mmol/l of ammonium sulphate. Working standards were prepared by diluting the stock standard 1 in 10 with distilled water. Aliquots of 0.25, 0.5, 0.75 and 1.0 ml of this solution were further diluted to 1 ml with water and 0.1 ml taken for analysis. These standards correspond to 330, 660, 990 and 1320 mU/l of enzyme catalytic activity concentration (cf. Procedure).

Samples

All blood samples were obtained from venous puncture allowed to clot and the serum removed and either assayed immediately or stored at −20 °C.

Procedure

To two tubes was pipetted 0.1 ml of serum and to one of the tubes was added 0.15 ml of substrate and to the second tube 0.15 ml of buffer. Both tubes were then capped and incubated in a water bath for 18 h at 22 °C. At the end of this time 1.5 ml of phenol reagent was added, mixed and 1.5 ml of hypochlorite solution added. The tubes were then incubated at 37 °C for 30 min and the blue colour read at 625 nm.

Standards for the calibration curve were prepared by the addition of 0.1 ml of the respective standards, 0.15 ml of buffer and 0.1 ml of a single serum sample, incubated at 22 °C for 18 h and subsequent treatment was as for the tests.

The ammonia content of the single serum used in all four standards for construction of the calibration curve was estimated by the addition of 0.15 ml of buffer to 0.1 ml of serum.

To construct the calibration curve the absorbance of the serum blank was deducted from the absorbance of each standard. The standards were equivalent to 330, 660, 990 and 1320 mU/l of enzyme activity. One unit of enzyme catalytic activity concentration (U/l) was defined as µmol of ammonia produced at 22 °C per minute per litre of serum.

The highest working standard (1320 mU/l) contained 1426 µmol/l of NH_4^+. The tubes were incubated for 18 hours (1080 min) and therefore the quantity of ammonia produced in µmol per minute per litre would be:

$$\frac{1426}{1080} = 1.320 \text{ U/l, corresponding to } 1320 \text{ mU/l.}$$

The enzyme catalytic activity concentrations of the test sera were obtained by deducting the absorbance of the serum blank from that of the test and reading the enzyme catalytic activity concentration from the calibration chart.

The 4 hour technique at 37 °C would be performed exactly as described above for 22 °C.

Optimization of the assay

The optimum conditions for the assay were determined using a pooled serum and varying one constituent while keeping all other constant.

Other enzyme assays

Alanine aminotransferase (37 °C) and γ-glutamyl transferase (37 °C) were assayed using the Boehringer Mannheim Diagnostic kits (Cat. No. 124954). The Technicon Instruments, SMA technique (method no. SF4-006FG5) was used for estimating the catalytic activity concentration of serum alkaline phosphatase (37 °C). Cytidine deaminase (22 °C) was assayed according to *Jones* et al. (4).

Results

pH-optimum

To determine the optimum pH for the assay, phosphate buffer ranging in pH from 5.5 to 8.0 at 0.5 pH intervals were used. The optimum pH for enzyme activity was 6.8 and therefore in the final procedure a pH of 6.8 was utilized. Figure 1 illustrates the results obtained.

Substrate

Varying concentrations of guanosine were used ranging from 0.7 mmol/l to 17.6 mmol/l. The optimum substrate concentration was found to be between 10 and 18 mmol/l as illustrated in figure 2. A concentration of 14.12 mmol/l was used throughout this work, since this gave a convenient quantity (40 mg) of substrate for weighing.

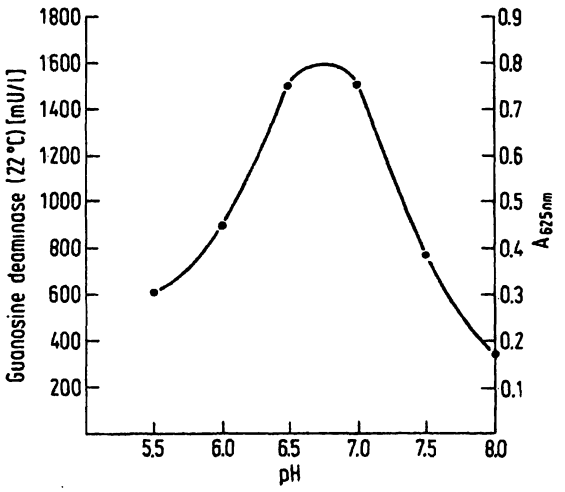

Fig. 1. Optimum pH for serum guanosine deaminase activity.

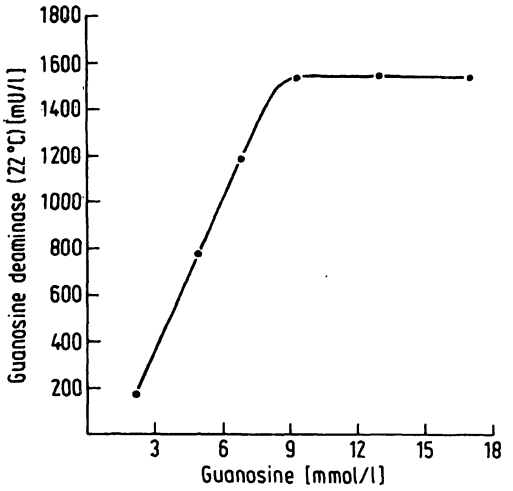

Fig. 2. Optimum substrate concentration for serum guanosine deaminase.

Linearity

The upper limit of enzyme activity at which a linear response could be evoked was determined using dilutions of a human serum from a patient possessing a very high level of enzyme catalytic activity concentration who was suffering from infective hepatitis. The value for this serum was 2300 mU/l. The serum was diluted with a serum containing normal catalytic concentration of guanosine deaminase (300 mU/l). Dilutions could not be made with buffer because of the quenching effect of serum protein on the *Berthelot* colour reaction. Figure 3 illustrates the results obtained and it is apparent that the technique described was linear up to 2300 mU/l of enzyme catalytic activity concentration.

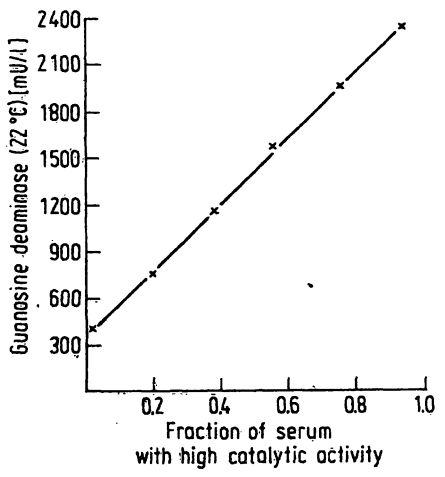

Fig. 3. Linearity of the method described for the estimation of serum guanosine deaminase at 22 °C. Values found when serum of high catalytic activity concentration (2300 mU/l) was diluted with a serum of normal catalytic activity concentration (300 mU/l).

Linearity of the method with time

The serum with 2300 mU/l of guanosine deaminase was estimated at two hourly intervals over a period of 18 h. A linear response was obtained throughout this period.

Influence of serum on the Berthelot reaction

Pooled serum was added to the standards since serum had a quenching effect upon the colour reaction. Varying volumes of phenol and hypochlorite reagents were added to 0.1 ml of serum in the presence of the highest working standard (= 1320 mU/l) and maximum colour development was obtained by the addition of 1.5 ml of phenol and hypochlorite solutions. A dialyzed ammonia free serum was used to investigate the quenching effect of protein. Varying the concentrations of the dialyzed serum between 40 g/l and 100 g/l had no effect upon the colour reaction when 1.5 ml of phenol and hypochlorite solutions were added. The addition of phenol and hypochlorite solutions in volumes below 1.0 ml decreased the absorbance. Although serum protein quenches the colour reaction the quenching is constant between a serum protein concentration of 40 g/l and 100 g/l. It is therefore for this reason that a single serum was added to the standards. A similar technique has been described by *Jones* et al. (4).

Reproducibility

When 20 replicates from a pooled human serum were placed within batches of samples and analysed on twenty consecutive days the mean value obtained

was 840 mU/l and the standard variation was 16 mU/l. Day to day reproducibility was maintained by estimating the activity of aliquots of a pooled serum stored at −20 °C.

'Reference range'

The sera from 100 'normal' patients were assayed to find a mean and 'reference range'. Care was taken in the selection of the sera to ensure that the patients did not have a disease. All of these sera were obtained from clinically healthy people attending the staff health department prior to employment as hospital staff. The mean, standard deviation and reference range for 18 h at 22 °C and 4 h at 37 °C are listed in table 1. The reference range was 'normally' distributed.

Tab. 1. Determination of mean and reference ranges of serum guanosine deaminase catalytic activity concentration.

	Incubation	
	18 hours at 22 °C	4 hours at 37 °C
Number of determinations	100	100
Mean guanosine deaminase values (mU/l)	220	600
Standard deviation (SD) (mU/l)	80	250
Reference range (± 2x SD) (mU/l)	60–380	100–1100

Serum guanosine deaminase in liver disorders

The serum guanosine deaminase catalytic activity concentrations among the various groups of patients are compared in figure 4. The catalytic concentration was increased markedly in hepatitis and in liver metastases. It did not change significantly in other liver disorders such as cirrhosis and obstructive jaundice.

Stability of guanosine deaminase activity

No detectable loss of enzyme activity was found when sera were stored at −20 °C for 14 days, while storage at 4 °C caused approximately a 10% loss of total activity in 3 days. Wherever possible fresh serum was used for routine measurement.

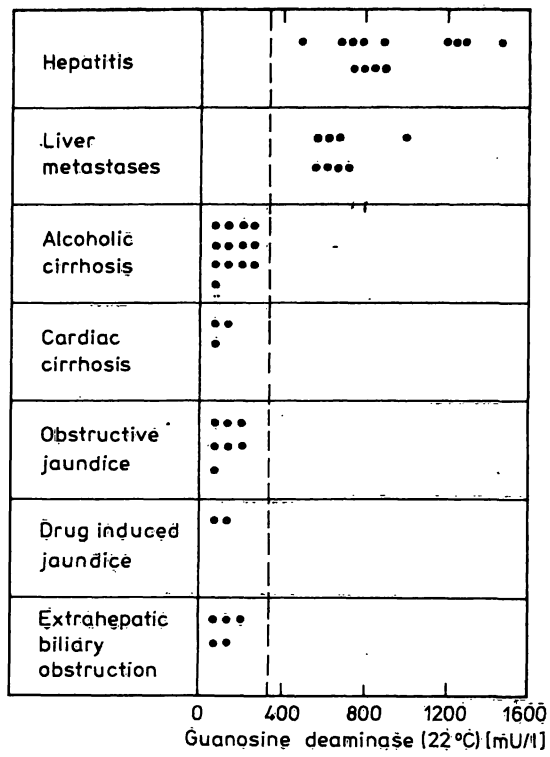

Fig. 4. Serum guanosine desaminase catalytic activity concentrations in various liver disorders. The line shows the normal range.

Comparison of guanosine deaminase with other tests of liver function

Of the enzymes investigated γ-glutamyltransferase was the most sensitive indicator of liver disease generally with 46 of the 51 sera tested having increased activity followed by alkaline phosphatase with 35 sera and alanine aminotransferase with 28.

In infective hepatitis and liver metastases guanosine deaminase was increased in all 21 patients (fig. 4), alkaline phosphatase in 18, γ-glutamyltransferase in 16 and alanine aminotransferase in 15. Figure 5 illustrates the results obtained for alanine aminotransferase and guanosine deaminase in a patient recovering from infective hepatitis.

Discussion

Guanosine deaminase is distinct from guanine deaminase (EC 3.5.4.15) which has no action on guanosine, adenosine or adenine (5, 6). Furthermore, guanine deaminase is found in liver and kidney (7).

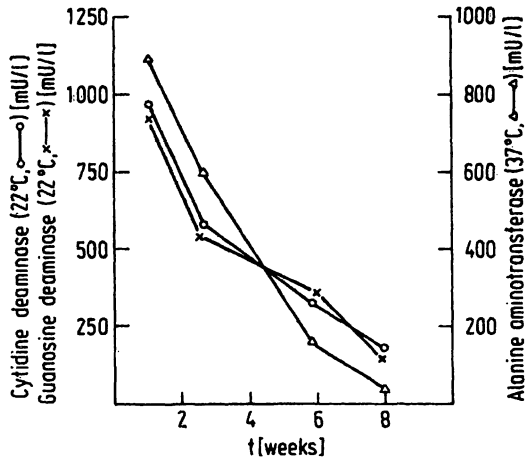

Fig. 5. Typical results found for serum cytidine deaminase (O——O), guanosine deaminase (x——x) and alanine aminotransferase (Δ——Δ) catalytic activity concentrations in a patient with infective hepatitis.

Guanine deaminase activity on the other hand has not been found in serum from patients suffering from kidney diseases (8).

This is the first report describing the clinical usefulness of estimating the serum activity of guanosine deaminase in liver disease.

Our preliminary findings suggest that serum guanosine deaminase catalytic activity concentration is increased in viral hepatitis and in patients with liver metastases but not in alcoholic cirrhosis, cardiac cirrhosis or obstructive jaundice. On the other hand, alanine aminotransferase, γ-glutamyltransferase and alkaline phosphatase were frequently increased in these diseases.

Serum cytidine deaminase catalytic activity concentration has been found by us to be increased in viral hepatitis but the estimation of guanosine deaminase has the advantage in that white blood cells contain a high activity of cytidine deaminase with no detectable guanosine deaminase activity.

The technique described for the estimation of guanosine deaminase is simple and inexpensive but requires an 18 h incubation period, although the 4 h technique at 37 °C described by us (9) for the estimation of cytidine deaminase can equally be applied to the estimation of guanosine deaminase in situations where an extremely urgent result is required. This 4 h technique utilizes the NADH-glutamate dehydrogenase reaction.

The technique described entails incubations at 22 °C for 18 h or 37 °C for 4 h. It was found impractical to use a temperature at 30 °C since at this temperature an incubation period of approximately 8 h would be required. An 8 h incubation period would not be suitable for use during the working day. In situations where the ambient laboratory temperatures are difficult to maintain at 22 °C then the 4 h at 37 °C would be the most suitable to use.

In the 4 h technique a temperature of 37 °C is used to obtain maximum ammonia production. The total time to complete this method would be approximately 6 h and would be unsuitable for use in a normal working day.

An incubation at 37 °C for 18 h would result in excess production of ammonia which would in turn cause non-linearity with poor reproducibility of results. Furthermore, at 37 °C bacterial growth could become a problem. Therefore an 18 h incubation at 22 °C as described by *Ressler* (10) for dCMP deaminase estimation was preferred.

The 18 h incubation at 22 °C has not in our experience caused problems in the growth of microorganisms which could have caused increased ammonia production. Although sterile glass tubes have been used for blood collection and sterile polystyrene reaction tubes used for the assays, aseptic precautions have not been taken.

Since the technique gives a linear response over the enzyme range 0—2300 mU/l direct measurement can be made over an extremely wide range. In our experience no patient serum has given guanosine deaminase values greater than 2450 mU/l.

Numerous enzymes have been proposed as sensitive indicators of liver disease but none have been found by us to be so discriminating as the deaminases. Furthermore, guanosine deaminase reflects the severity of the disease.

Measurement of the rate of production of ammonia offers a simple means of detecting guanosine deaminase and is ideally suited for incorporation into a liver function profile. A detailed clinical evaluation of serum guanosine deaminase together with a multicentre evaluation of the enzyme is at present taking place.

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